Enzyme Mechanisms

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Thioester Hydrolysis and C–C Bond Formation by Carboxymethylproline Synthase from the Crotonase Superfamily**

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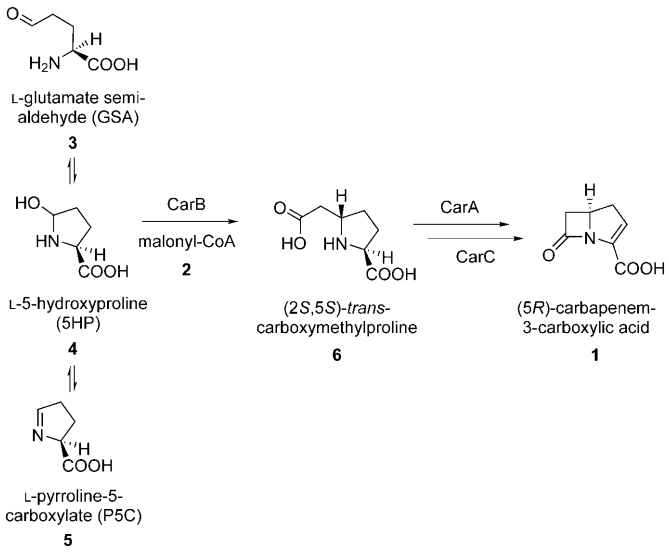
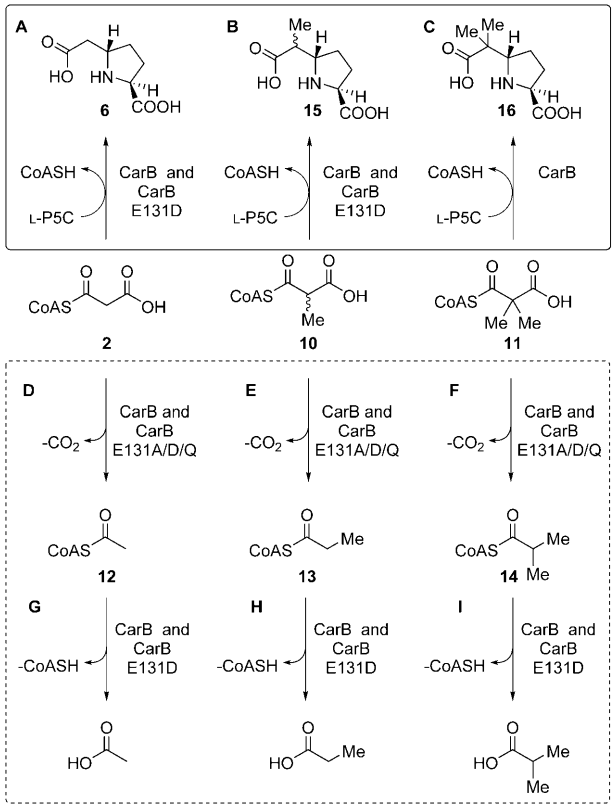
Carboxymethylproline synthase (CarB), a member of the crotonase superfamily (CS) of enzymes,[1] catalyzes the committed step in the biosynthesis of (5*R*)-carbapenem-3-carboxylic acid (**1**; Scheme 1),[2] the simplest of the medicinally important carbapenem antibiotics.[3,4] CarB mediates the reaction of malonyl-coenzyme A (**2**) with L-glutamate semialdehyde (GSA, **3**, which exists in equilibrium with L-5-hydroxyproline (5HP, **4**) and L-pyrroline-5-carboxylate (P5C, **5**), collectively GHP) to give, after thioester hydrolysis, (2*S*,5*S*)-*trans*-carboxymethylproline (*t*-CMP, **6**; Scheme 1).[2,5] CarB is unusual amongst CS enzymes because, in addition to decarboxylation and thioester hydrolysis steps, it catalyzes C–C bond formation leading to a substituted heterocycle (Scheme 2, reactions A–C).



Scheme 1. Biosynthesis of (5*R*)-carbapenem-3-carboxylic acid (**1**) in *Pectobacterium carotovorum* showing the reaction of wild-type CarB.



Scheme 2. Reactions catalyzed by wild-type CarB and CarB variants. Reactions A–C (solid box) are in the presence of GHP; reactions D–I (dashed box) are in the absence of GHP.

CarB catalysis is proposed to proceed by decarboxylation of **2** to generate an enolate, which is stabilized by hydrogen bonding in a conserved oxy-anion hole[6] (Figure 1 and Figure S1 in the Supporting Information). C–C bond formation can then occur, either by reaction of the enolate with **5** or by aldol reaction of the enolate with **3** (followed by elimination of water and conjugate addition, Scheme 3). The observation of *t*-CMP-CoA (**7**) by mass spectrometry

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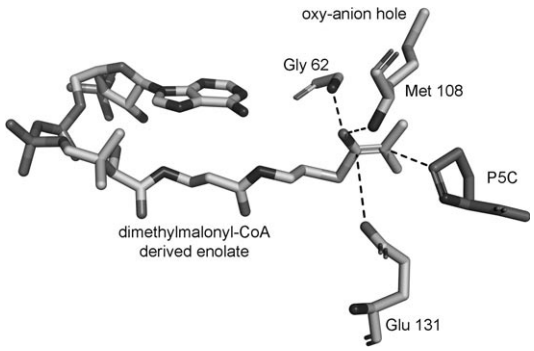
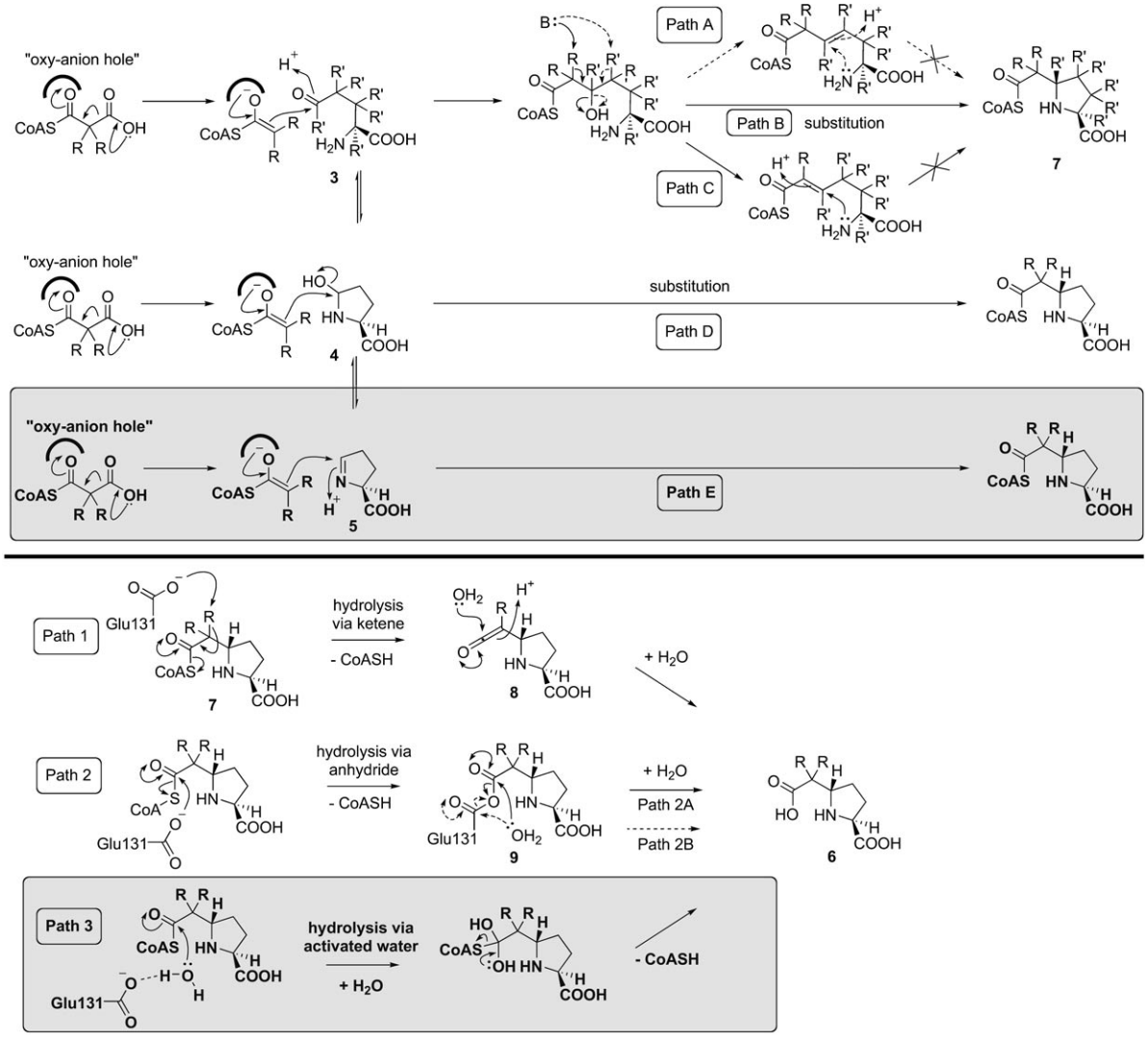


Figure 1. View from a CarB crystal structure[6] with the dimethylmalonyl-CoA derived enolate and P5C (**5**) modeled into the active site (see the Supporting Information). The position of the dimethylmalonyl-CoA derived enolate was fixed by residues forming the oxy-anion hole (Gly 62 and Met 108). Glu 131 is suitably positioned (≈4 Å from the thioester carbonyl) to activate a water molecule.

(MS)[5] implies that C–C bond formation precedes thioester hydrolysis.

Despite the central importance of thioesters in biology, there are relatively few studies on their hydrolysis mechanisms.[7] Proposed mechanisms include those involving acyl–enzyme complexes (e.g. in fatty acid synthase[8] and some polyketide thioesterases),[9] although other possibilities, including those involving ketene intermediates, have been considered.[10] Within the ubiquitous crotonase superfamily, the mechanisms of CoA thioester hydrolysis are uncertain. Wong and Gerlt[11] have proposed a mechanism for hydroxyisobutyryl-CoA hydrolase (HICH) involving a mixed-anhydride intermediate, formed by reaction of the substrate CoA thioester with the Glu 143 side chain, followed by hydrolysis of the anhydride at the enzyme-derived carbonyl. For 4-chlorobenzoyl-CoA dehalogenase, a related mechanism involving an ester linkage formed between an aspartyl residue and the aromatic ring of the substrate has been proposed.[12]

Scheme 3 shows plausible mechanisms for C–C bond formation and thioester hydrolysis steps in CarB catalysis. For hydrolysis, mechanisms involving a ketene (**8**, path 1) or an anhydride (**9**, path 2) or direct attack of water at the carbonyl



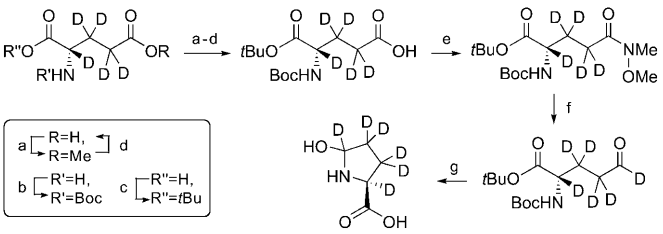
Scheme 3. CarB mechanisms. Paths A–E: possible paths leading to the formation of *t*-CMP-CoA (**7**). Paths 1–3: possible hydrolysis mechanisms to yield *t*-CMP (**6**, see text for discussion). Gray boxes: mechanistic paths identified as most likely by this work. The dark curve indicates the enzyme "oxy-anion hole". R = H or CH_3, R′ = H or ^2H.

(path 3) are all reasonable. The ketene mechanism is relevant because of the propensity of CS enzymes to form enolates,[1,11] and the likely involvement of an enolate in CarB-catalyzed C–C bond formation. Here we report mechanistic studies on the C–C bond-forming and thioester hydrolysis steps of CarB.

Initially we investigated the role of the Glu131 residue in CarB in catalysis by producing and analyzing Glu131 A/D/Q variants. The E131 A/Q variants did not catalyze production of **6** or **7** from **2** and GHP but did catalyze decarboxylation of **2**, methylmalonyl-CoA (**10**), and dimethylmalonyl-CoA (**11**) to acetyl-CoA (**12**), propionyl-CoA (**13**), and isobutyryl-CoA (**14**), respectively (Scheme 2). In contrast, the more conservative E131D variant catalyzed production of **6** from **2** and GHP, with a much lower (≈5%) specific activity for decarboxylation than wild-type (wt) CarB (see Table S1 and Figure S2 in the Supporting Information). Interestingly, for both wt CarB and CarB E131D, GHP stimulated decarboxylation of **2** but inhibited the decarboxylation with CarB E131A and E131Q. Both wt CarB and CarB E131D also catalyzed hydrolysis of **12**–**14** but variants E131A/Q did not. Overall these results reveal that Glu131 is important in both C–C bond formation and thioester hydrolysis steps but does not play an essential role in decarboxylation.

We then confirmed the reported[2,5] production of C6 epimers of 6-methyl-*t*-CMP (**15**) from **10** and GHP in an approximate 1:1 ratio (determined by NMR spectroscopy to be (2*S*,5*S*,6*R*)/(2*S*,5*S*,6*S*); 11:9 ratio for wt CarB (see Figure S3 in the Supporting Information)) by LC–MS and ^{1}H NMR analyses; the assigned stereochemistry was based on analysis of the ^{1}H–^{1}H coupling constants and nuclear Overhauser enhancement studies. Because successful conversion of a dialkylmalonyl-CoA derivative to a 6,6′-dialkyl-*t*-CMP by CarB cannot occur by mechanisms involving either a ketene intermediate or conjugate addition,[7] **11**[13] (Figure S4 in the Supporting Information) was tested as a substrate. LC–MS analyses implied the formation of 6,6′-dimethyl-CMP (Figure S5 in the Supporting Information; top: *m*/*z* 202 Da, $[M+H]^+$). After incubation on a larger scale and semipreparative LC–MS, sufficient product was obtained for NMR analyses (≈50 μg), which revealed the production of (2*S*,5*S*)-6,6′-dimethyl-*t*-CMP (**16**, Figure 2, Figure S5 in the Supporting Information). Of the Glu131 variants, only CarB E131D catalyzed formation of **15** from methylmalonyl-CoA; none of the variants catalyzed formation of **16**. Assuming the mechanism is the same for **2** and **11**, these observations eliminate both a conjugate addition process for heterocycle formation (Scheme 3, path C) and thioester hydrolysis occurring via ketene **8** (Scheme 3, path 1).

To eliminate other condensation mechanisms for the formation of **16** involving exchange of hydrogen on GHP (i.e. elimination of water from C4/C5 before addition; Scheme 3, path A), we then prepared [$^2H_6$]GHP (Scheme 4). Introduction of the deuterium label at C5 was achieved by reaction of deuterated Schwartz reagent ([Cp_2Zr^2HCl]) with a suitably protected *N*,*O*-dimethylhydroxylamine glutamate derivative (Scheme 4).[14] Deprotection with 10% aqueous (^2H_2O) deuterated formic acid ($^2HCO_2{}^2H$) to avoid loss of label at C4 of GHP gave [$^2H_6$]GHP. The product of incubating [$^2H_6$]GHP with wt CarB and **11** was 6,6′-dimethyl-*t*-CMP (**17**), fully (>90% incorporation by MS and NMR) deuterated at the C2, C3, C4, and C5 positions (Figure 2, top and Figure S5 in the Supporting Information; *m*/*z* 208 Da $[M+H]^+$). This observation eliminates a condensation mechanism involving C4 desaturation (Scheme 3, path A).



Scheme 4. Synthesis of [$^2H_6$]GHP: a) $SOCl_2$, MeOH; b) $(Boc)_2O$, NEt_3, THF; c) *t*BuOH, dimethylaminopyridine, *N*,*N*′-dicyclohexylcarbodiimide, CH_2Cl_2; d) LiO^2H, THF/^2H_2O (1:1), 26% yield over four steps; e) HN(Me)OMe, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate, NEt_3, CH_2Cl_2, 82%; f) [Cp_2Zr^2HCl], THF, 66%; g) 10% $^2HCO_2{}^2H$ in ^2H_2O. Boc = *tert*-butoxycarbonyl.

To investigate the possibility of thioester hydrolysis proceeding via a mixed-anhydride intermediate (Scheme 3, path 2), incubations in buffered $H_2{}^{18}O$ ($H_2{}^{18}O/H_2{}^{16}O$; 7:1) were carried out. As with **2**,[6] incubations with **11** led to the incorporation of a single ^{18}O into the 6,6′-dimethyl-*t*-CMP product (≈70% by LC–MS, *m*/*z* 204 Da $[M+H]^+$ for ^{18}O-labeled material, *m*/*z* 202 Da $[M+H]^+$ for ^{16}O-labeled material, Figures S6–S8 in the Supporting Information). Trypsin digest analyses were then performed on wt CarB and CarB E131D from the same assays used for LC–MS analyses (which had been incubated with GHP and **11** or **2**) to test for exchange into Glu131/Asp131. The mass of the peptide fragment corresponding to residues 120–133 was identical in the samples from experiments with both the labeled ($H_2{}^{18}O$) water and the unlabeled ($H_2{}^{16}O$) water; the lack of ^{18}O incorporation from solvent into the side chains of Glu131 (wt CarB) or Asp131 (E131D CarB variant) eliminates path 2B (Scheme 2) and demonstrates that thioester hydrolysis occurs by a different mechanism than that proposed for HICH.[11]

Scheme 3 summarizes mechanistic possibilities for the formation of the *t*-CMP thioester. At pH 8.0, the imine form of GHP, **5**, is preferred.[15] Reaction of the thioester enolate

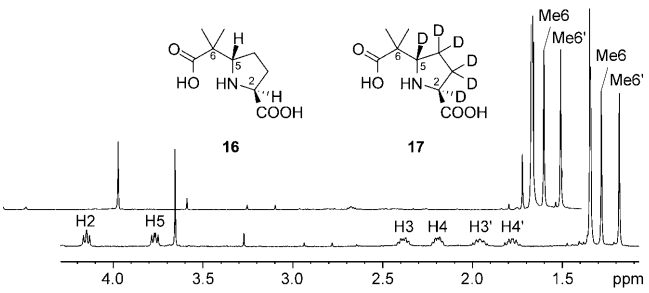


Figure 2. ^{1}H NMR spectra of protonated (**16**; bottom) and deuterated (**17**; top) products from incubations of dimethylmalonyl-CoA **11** with GHP and [$^2H_6$]GHP, respectively. Peaks at $\delta = 3.65$ and 1.35 ppm correspond to Tris and dimethylmalonic acid, respectively.

directly with **4** by S_N2 reaction (Scheme 3, path D) seems unlikely, in part because **4** exists as a mixture of C5 hemiaminal epimers that can readily eliminate water to form protonated **5** likely giving a lower energy reaction path (Scheme 3, path E). Further, an S_N2-type mechanism would have to occur on only one of the two epimeric alcohol forms of **4** to produce **6** exclusively, as is observed.[6]

The formation of **16** eliminates the possibility of a conjugate addition mechanism (Scheme 3, path C). The labeling studies eliminate path A. Path B cannot be entirely discounted, but it seems likely that path E, involving **5** (S_N1-type reaction), is the most probable mechanism for formation of *t*-CMP-CoA intermediates. The observation that the hindered dimethylmalonyl-CoA reacts at all is also indirect evidence for this mechanism because the iminium ion route (path E) is less sterically demanding than an S_N2 route (path B).

Three plausible hydrolysis mechanisms are shown in Scheme 3 (paths 1–3). An acyl-cation mechanism (not shown) is unlikely, given the role of the highly conserved oxy-anion hole in CS catalysis,[1] whilst the observation that **11** is converted into **16** rules out the ketene path (path 1). The mechanism proceeding by hydrolytic attack on the carbonyl of an anhydride intermediate derived from residue Glu131 (path 2B) is eliminated by ^{18}O-labeling experiments. We cannot entirely rule out the anhydride mechanism involving hydrolytic attack on the *t*-CMP-derived carbonyl (path 2A), but the available evidence points against it because, at least with the sterically hindered 6,6′-dimethyl-*t*-CMP intermediate, this path requires hydrolysis adjacent to a sterically hindered quaternary carbon atom.

We conclude that C–C bond formation by CarB most probably occurs by reaction of P5C with an oxy-anion-stabilized enolate, and thioester hydrolysis most probably proceeds by direct attack of an activated water molecule (Scheme 3, paths E and 3, gray boxes). The results may have implications for other CS family members and enzyme-catalyzed thioester hydrolysis in general.

Experimental Section

CarB purification and incubation: Wild-type CarB and CarB variants were prepared and assayed as reported,[2] except that glycerol was omitted from all buffers and final desalting was into 50 mM Tris-HCl pH 7.5. Assays were analyzed by LC–MS, HPLC, or both (see the Supporting Information for more details).

Trypsin digestion of CarB was performed as reported[2] using "Sequencing Grade Modified Trypsin" (Promega, UK) with minor modifications (see the Supporting Information for the full procedure) before peptide fragments were analyzed by MS–MS.

The specific activities were determined by real-time NMR monitoring of CarB-catalyzed reactions under the reported conditions,[2] except that 100 mM CoA stock solutions in H_2O were diluted to the appropriate concentration with ^2H_2O and 600 mM deuterated [$^2H_{11}$]Tris (adjusted to p^{2}H 9.0 with ^{2}HCl) in ^2H_2O buffer was used; assays were scaled up to a final volume of 75 μL before analysis at 37 °C using a Bruker AVIII 700 MHz spectrometer (with an inverse cryoprobe optimized for ^{1}H observation).

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